REMARKS

The remainder of this Amendment is set forth under appropriate subheadings for the convenience of the Examiner.

I. Restriction of Species

The Examiner has withdrawn the restriction requirement set forth in the Office Action mailed on September 27, 2001 in view of a restriction set forth in Interview Summary, Paper No. 10, faxed to applicant's attorney on March 4, 2002.

Applicants elect to prosecute the invention of Species II, drawn to inhibitor OM99-2 and a method of making an intermediate for synthesis of OM99-2. Claims as amended herein that are readable on the elected species are Claims 10, 12-14, 16,17, and 20-27. Applicant reserves the right to file a continuing application or take such other appropriate action as deemed necessary to protect the non-elected inventions. Applicant does not hereby abandon or waive any rights in the non-elected inventions.

II. Amendments to the Specification

Applicants have amended the specification on page 46, lines 19, 20 and 31 to indicate that Resource-Q is a trademark and to reference a Pharmacia catalog that describes the product.

Applicants have amended the specification on page 19, line 10 to replace the formula "CH_2OOH" with the formula "CH_2COOH". Support for this amendment can be found in the parenthetical statement following the formula on page 19, lines 10-11 indicating that the formula is the side chain of aspartic acid.

Applicants have also amended the specification on page 19, lines 6-7 to indicate that the amino acid on the substrate that is most determinative of binding to memapsin 2 is at P_1' not S1'. Support for this amendment can be found on page 15, lines 1-8 of the specification which indicates that the nomenclature for this substrate residues is P_1', not S1', which is a subsite on memapsin 2.

III. Claim Amendments

Applicants have cancelled Claims 1-3, 5-9, 11, 15, 19 and 19.

Applicants have redrafted Claim 5 as new Claim 24. In Claim 24, Applicants claim a compound the includes the structural formula of OM99-2 and pharmaceutically acceptable salts thereof. Support for this amendment can be found in Figure 3B and on page 7, lines 25-26 and page 27, lines 1-9 of the specification.

Applicants have amended Claims 10, 12, 16 and 17 to depend from new Claim 24, and Claim 13 to depend from Claim 12. Claims 10, 12, 13 and 14 have also been amended to indicate that the claimed compounds inhibit memapsin 2.

Applicants have amended Claim 20 to include the method steps of the claimed method of preparing a Leu*Ala isostere. In addition, Applicants have added Claims 25-27 which are dependent from Claim 20. Claim 25 is directed to a method of preparing N-(tert-butoxy-carbonyl)-leucinal, which is used as a starting material in the preparation of Leu*Ala isostere, and Claims 26 and 27 are directed to Leu*Ala isosteres having different protecting groups. Support for the amendments to Claim 20 and for new Claims 25-27 can be found in Scheme 1 on page 37 and Scheme 2 on page 38 of the specification and in the description on page 37, line 1 to page 44, line 7 of the specification.

Claim 23 has been amended to replace the acronym "APP" with the phrase 'amyloid precursor protein." Support for this amendment can be found on page 2, lines 4-6 of the specification.

IV. Objection to Improper Use of Trademark

The Examiner objects to the use of a trademark to designate the type of FPLC column used to purify the active fraction of memapsin 2 on page 46, lines 19, 20, and 31 of the specification. The Examiner also indicates that the specification should be amended to include published product information sufficient to show that the generic terminology or description is inherent in the article referred to by the trademark.

Applicants have amended the specification to indicate that the term "Resource-Q" is a trademark and to include a reference to a Pharmacia catalogue that describes the product.

V. Rejection of Claims 1-3, 5, 10-17 and 20-23 Under 35 U.S.C. §112, First Paragraph

The Examiner states that the specification is enabling for inhibition of memapsin 2 by OM99-1 and OM99-2 inhibitors and use of OM99-1 and OM99-2 in designing, synthesizing and testing the inhibitory activity towards memapsin 2 *in vitro*. However, the Examiner states that the specification does not provide enablement for a product defined by reference to a desirable characteristic or property. The Examiner also states that the specification does not enable a method of diagnosing and treating a patient for Alzheimer's disease, and that the state of the art is unpredictable and sufficiently complex so that a person of skill in the art would not accept the claimed method of treating or preventing Alzheimer's disease as obviously valid and correct without demonstration of evidence or data.

Applicants have canceled Claims 1-3, thereby obviating the rejection with regard to these claims.

Applicants have redrafted Claim 5 as new Claim 24. Claim 24 claims a compound having a structural formula that comprises the structure of OM99-2 and pharmaceutically acceptable salts thereof. Applicants have shown how to prepare OM99-2 in Example 7, on page 36, line 1 to page 44, line 11 of the specification. In addition, Applicants have demonstrated that OM99-2 inhibits memapsin 2 in Example 8 on page 44, lines 13-18 of the specification. New Claim 24 meets the requirements of 35 U.S.C. § 112, first paragraph.

Applicants have amended Claims 10, 12, 13, 14, 16 and 17 to depend from Claim 24. Claims 10, 12, 13 and 14 are directed to compounds that have K_i of less than or equal to 10^{-7} M, 10^{-6} M, 2 nM and 1 nM, respectively, for memapsin 2. Applicants have shown that OM99-2 has a K_i of 9.58×10^{-9} M in Figure 5B and on page 44, line 18 of the specification. Applicants disclose how to make compounds that inhibit memapsin 2 in the claimed K_i range for Claims 10, 12 and 13 and near the claimed K_i range for Claim 14. Therefore, Claims 10, 12, 13, and 14 meet the requirements of 35 U.S.C. § 112, first paragraph.

Claim 16, as amended, is directed to compounds of Claim 24 that are permeable to the blood-brain barrier, and Claim 17, as amended, is directed to compounds of Claim 24 that block the cleavage of memapsin 2 under physiological conditions. Applicants have indicated that, for a compound to be permeable to the blood-brain barrier, preferably, it has a molecular weight of 800 daltons or less (page 55, lines 15-18 of the specification), and it is composed of hydrophobic

amino acid residues (page 36, lines 9-12 of the specification). Therefore, Applicants have disclosed how to make compounds that are permeable to the blood-brain barrier. Compounds that are permeable to the blood-brain barrier will be able to penetrate tissues in the body where memapsin 2 is found. In addition, compounds of Claim 24 have a transition state isostere at the peptide bond that is cleaved by memapsin 2 rather than at an amide bond. The transition state isostere has the geometry of the transition state which has a higher affinity for the enzyme than the substrate because the enzyme active site stabilizes the transition state (page 16, lines 1-4 of the specification). Therefore, compounds of Claim 24 that include the amino acid sequence of OM99-2 which is recognized by memapsin 2, but have a transition state isostere at the cleavage site, should have a higher affinity for the enzyme than the natural substrate. Therefore, they will block cleavage of amyloid precursor protein (APP) by memapsin 2 under physiological conditions.

Claim 20, as amended, includes method steps for preparing a Leu*Ala dipeptide isostere. Applicants have disclosed the details of this method in Example 7 on page 37, line 1 to page 44, line 7 of the specification such that a person of ordinary skill in the art could prepare the Leu*Ala isostere. Therefore, Claim 20, as amended, is enabled.

Claim 21 is directed to a method of treating, stopping the progression of or decreasing the likelihood of developing Alzheimer's disease, not a method of diagnosing a patient with Alzheimer's disease, as stated by the Examiner. Applicants have disclosed that memapsin 2 is an enzyme primarily found in the brain and that it recognizes a specific sequence in APP and cleaves it to form amyloid β-protein (page 14, lines 8-16 of the specification). Amyloid β-protein is a major constituent of the plaques found in high concentrations in the brains of Alzheimer's patients. Amyloid β-protein has been shown to be toxic to nerve cells because it increases the calcium influx mediated by glutamate to toxic levels (Exhibit A: Science News (1993), 141:152-153, p. 153, Col. 3, paragraphs 2 and 3). Researchers have shown that one form of early onset, or familial, Alzheimer's disease is caused by a mutation in one amino acid of APP in the cleavage site that is recognized by memapsin 2 (Exhibit A, page 152, Col. 3, lines 2-9). This causes more accumulation of amyloid β-protein in the brains of patients with early onset Alzheimer's disease than in the brains of patients without the mutation. Applicants have shown that memapsin 2 cleaves the mutated sequence at a higher rate than the wild type sequence of

APP (page 14, lines 12-13 of the specification) indicating that increased rate of cleavage of the mutated APP sequence is responsible for the early accumulation of amyloid β-protein, and thus early onset of Alzheimer's disease, in patients with the mutated APP sequence. Non-familial Alzheimer's disease typically has a later onset than familial Alzheimer's disease, but also results in the development of plaques composed mainly of amyloid β-protein. Since memapsin 2 has been shown to be responsible for the production of amyloid β-protein and increased activity of memapsin 2 has been shown to increase the accumulation of amyloid β-protein and cause early onset of Alzheimer's disease, decreasing in the activity of memapsin 2 will reduce the accumulation of amyloid β-protein thereby reducing the likelihood of developing Alzheimer's disease or halting further progression of the disease in patients who already have the disease. Applicants have established a nexus between compounds that include the structural formula of OM99-2 and the treatment or prevention of Alzheimer's disease by showing that these compounds inhibit the activity of memapsin 2, and thus, inhibit the production of amyloid β-protein. Moreover, animal models are available which simulate the pathology of Alzheimer's disease. For example, mice that have been genetically engineered to over express APP exhibit significant Alzheimer's disease pathology (Exhibit B: Alzheimer's Disease Review 2 (1997), 2-10, page 38, Col. 2, Conclusions). Thus, a person of ordinary skill in the art could readily test a compound that included the structural formula of OM99-2 in animal models to determine whether it reduced the accumulation of amyloid β-protein.

Moreover, the Court of Appeals has stated that:

> When rejecting a claim under the enablement requirement of section 112, the PTO bears an initial burden of setting forth a reasonable explanation as to why it believes that the scope of protection provided by that claim is not adequately enabled by the description of the invention provided in the specification of the application; this includes, of course, providing sufficient reasons for doubting any assertion in the specification as to the scope of enablement. *In re Wright*, 27 U.S.P.Q. 1510, 1513 (Fed. Cir. 1993).

The Examiner states that there is a paucity of understanding concerning the mechanism of amyloid formation in Alzheimer's disease and cites Cordell, *et al.*, U.S. Patent No. 5,221,607 (hereinafter "'607") in support of this statement. However, the instant specification provides evidence that addresses this paucity of understanding by showing that memapsin 2 is involved in cleaving APP to form amyloid β-protein and that increased cleavage of APP by memapsin 2, as in familial Alzheimer's disease, results in early onset of the disease. In addition, '607 indicates that the frequency of neuritic plaques in Alzheimer's patients correlates with the degree of dementia ('607, Col. 1, lines 55-58) and that therapeutics that treat amyloidosis are useful in the treatment of Alzheimer's disease ('607, Col. 2, lines 3-5). Thus, '607 provides further support for Applicants' assertion that the compounds of the invention, which inhibit the activity of memapsin 2 and thereby inhibit the formation of amyloid β-protein, are useful in treating Alzheimer's disease.

In discussing the requirements for a valid 35 U.S.C. § 112, first paragraph rejection the Court of Customs and Patent Appeals has stated that:

> As a matter of Patent Office practice, then, a specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented *must* be taken as in compliance with the enabling requirement of the first paragraph of § 112, *unless* there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support. *In re Marzocchi and Horton*, 169 U.S.P.Q. 367, 369 (C.C.P.A. 1971) (emphasis in the original).

The Examiner has not provided any evidence to rebut Applicants' evidence that memapsin 2 is involved in the formation of amyloid β-protein and that increased memapsin 2 activity, as in familial Alzheimer's disease, results in increased amyloid β-protein formation and the early onset of Alzheimer's disease. In addition, the Examiner has provided no reason to doubt Applicants' evidence that the compounds of the invention inhibit memapsin 2, and thus, inhibit the formation of amyloid β-protein. Since Applicants have established a nexus between increase cleavage of

APP memapsin 2 and the early onset of Alzheimer's disease, and animal models are readily available to test the activity of inhibitors of memapsin 2, Applicants' claimed method of treating or decreasing the likelihood of getting Alzheimer's disease meet the requirements of 35 U.S.C. § 112, first paragraph. Therefore, Applicants respectfully request that the rejection be reconsidered and withdrawn.

VI. Rejection of Claims 1-3, 5, 10-17 and 20-23 Under 35 U.S.C. §112, Second Paragraph

The Examiner rejects Claims 1-3, 5 and 10 because they fail to recite a function or use of the inhibitors. In addition, the Examiner rejects Claim 3 because the recitation of "L0-" and "P1'" is indefinite. Applicants have cancelled Claims 1-3 and 5 obviating the rejection with regard to these claims. Applicants have amended Claim 10 to indicate that the compounds inhibit memapsin 2.

The Examiner rejects Claims 11 and 21 because they refer to Table 2 in the specification and the Examiner states that referring back to a table in the specification is unacceptable claim language. Applicants have cancelled Claim 11 and have amended Claim 21 to remove the reference to Table 2.

The Examiner rejects Claim 15 because there is insufficient antecedent basis for the recitation "amino acids 18-379 of memapsin 2." Applicants have cancelled Claim 15.

The Examiner rejects Claim 20 because a method of preparing Leu*Ala is claimed but no method steps are recited. Applicants have amended Claim 20 to recite method steps.

The Examiner rejects Claim 21 because the claim recites the phrase "administering to the individual an effective amount." The Examiner cannot ascertain what is meant by the term "effective amount" because the Examiner cannot find any amount of inhibitor claimed or disclosed.

M.P.E.P. § 2173.05(c)(III) states that the phrase "effective amount" in a claim is definite when read in light of the supporting disclosure and in the absence of any prior art which would give rise to uncertainty about the scope of the claim. In the instant case, Applicants have indicated that the appropriate dosage to be administered to an individual is a dosage which decreases the symptoms of Alzheimer's disease, such as decreasing the amount of APP cleavage

which could be measured by measuring the amount of the cleavage product, amyloid β-protein in the individual (page 27, lines 16-21 of the specification).

The Board of Patent Appeals and Interferences in deciding that the term "effective amount" in a pharmaceutical composition claim and method of treatment claims was definite stated that:

> As is well known, the specific dosage for a given patient under specific conditions and for a specific disease will routinely vary, but determination of the optimum amount in each case can readily be accomplished by simple routine procedures. See *Ex parte Skuballa,* 12 U.S.P.Q.2d 1570, 1571 (B.P.A.I. 1989).

Since Applicants have disclosed in the specification the results to be achieved by administering a therapeutically effective amount of a compound of the invention, a person of ordinary skill in the art would be able to determine specific dosages with only routine experimentation. Thus, Claim 2, as amended, meets the requirements of 35 U.S.C. § 112, second paragraph. Therefore, Applicants respectfully request that the rejection be reconsidered and withdrawn.

The Examiner rejects Claim 23 because the acronym "APP" is used. Applicants have amended Claim 23 to replace the acronym with the phrase "amyloid precursor protein."

VII. Rejection of Claims 1-2, 10 and 16-17 Under 35 U.S.C. § 102(b) Over Chrysler, *et al.*, U.S. Patent No. 5,744,346 (Hereinafter "'346")

The Examiner rejects Claims 1-2, 10 and 16-17 under 35 U.S.C. § 102(b) over '346 because '346 discloses a memapsin 2 inhibitor which binds to the active site of memapsin 2 and inhibits proteolytic cleavage of APP.

Applicants have cancelled Claims 1, 2, 16 and 17, thereby obviating the rejection in regard to these claims.

Applicants have amended Claim 10 so that it depends from new Claim 24 and therefore, incorporates the limitations of Claim 24. Claim 24 is directed to compounds that include the structural formula of OM99-2. '346 does not disclose any compounds that include the structural

formula of OM99-2. Therefore, Claim 10, as amended, is not anticipated by '346, and Applicants respectfully request that the rejection be reconsidered and withdrawn.

SUMMARY AND CONCLUSIONS

Applicants have shown how to make and use compounds that include the structure of OM99-2. Therefore, Claims 10, 12-14, 16 and 17 which, as amended, depend from new Claim 24 meet the requirements of 35 U.S.C. § 112, first paragraph. In addition, Applicants have demonstrated a nexus between the rate of cleavage of APP and the onset of Alzheimer's disease and have shown that compounds that include the structure of OM99-2 inhibit APP cleavage by memapsin 2 because they have a higher affinity for memapsin 2 than APP. Therefore, Claim 21 meets the requirements of 35 U.S.C. § 112, first paragraph.

In view of the above amendments and remarks, it is believed that all claims are in condition for allowance, and it is respectfully requested that the application be passed to issue. If the Examiner feels that a telephone conference would expedite prosecution of this case, the Examiner is invited to call the undersigned at (978) 341-0036.

Respectfully submitted,

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Dated: 12/17/02

MARKED UP VERSION OF AMENDMENTS

Specification Amendments Under 37 C.F.R. § 1.121(b)(1)(iii)

Replace the paragraph at page 19, lines 5 through 12 with the below paragraph marked up by way of bracketing and underlining to show the changes relative to the previous version of the paragraph.

(1) The primary specificity site for a memapsin 2 substrate is [subsite] position, P_1'. This means that the most important determinant for substrate specificity in memapsin 2 is the amino acid[, S1'] at P_1'. P_1' must [contain] be a small side chain for memapsin 2 to recognize the substrate. Preferred embodiments are substrate analogs where R_1 of the P_1' position is either H (side chain of glycine), CH_3 (side chain of alanine), CH_2OH (side chain of serine), or [CH_2OOH] CH_2COOH (side chain of aspartic acid). Embodiments that have an R1 structurally smaller than CH_3 (side chain of alanine) or CH_2OH (side chain of serine) are also preferred.

Replace the paragraph at page 46, lines 12 through 27 with the below paragraph marked up by way of bracketing and underlining to show the changes relative to the previous version of the paragraph.

This memapsin 2 solution was allowed to stand at 4 °C for 2-3 weeks. The total volume of approximately 16 liters was concentrated to 40 mls using ultra-filtration (Millipore) and stir-cells (Amicon), and centrifuged at 140,000 xg at 30 minutes in a rotor [pre-equilbrated] pre-equilibrated to 4 °C. The recovered supernatant was applied to a 2.5 x 100 cm column of S-300 equilibrated in 0.4 M urea, 20 mM Tris-HCl, pH 8.0, and eluted with the same buffer at 30 ml/hour. The active fraction of memapsin 2 was pooled and further purified in FPLC using a 1 ml [Resource-Q] Resource-Q® (Pharmacia

Biotech 1997, page 195) column. Sample was filtered, and applied to the [Resource-Q] Resource-Q® column equilibrated in 0.4 M urea, 50 mM Tris-HCl, pH 8.0. Sample was eluted with a gradient of 0 - 1 M NaCl in the same buffer, over 30 ml at 2 ml/min. The eluents containing memapsin 2 appeared near 0.4 M NaCl which was pooled for crystallization procedure at a concentration near 5 mg/ml.

Replace the paragraph at page 46, line 28 through page 47, line 3 with the below paragraph marked up by way of bracketing and underlining to show the changes relative to the previous version of the paragraph.

The activation of the folded pro-enzyme to mature enzyme, memapsin 2, was carried out as described above, i.e., incubation in 0.1 M sodium acetate pH 4.0 for 16 hours at 22 °C. Activated enzyme was further purified using anion-exchange column chromatography on [Resource-Q] Resource-Q® anion exchange column. The purity of the enzyme was demonstrated by SDS-gel electrophoresis. At each step of the purification, the specific activity of the enzyme was assayed as described above to ensure the activity of the enzyme.

Claim Amendments Under 37 C.F.R. § 1.121(c)(1)(ii)

10. (Amended) The [inhibitor] compound of claim [1] 24 having [an] a K_i of less than or equal to 10^{-7} M for memapsin 2.

12. (Amended) The [inhibitor] compound of claim [11] 24 having a K_i of less than or equal to 10^{-6} M for memapsin 2.

13. (Amended) The [inhibitor] compound of claim [11] 12 having a K_i of less than or equal to 2 nM for memapsin 2.

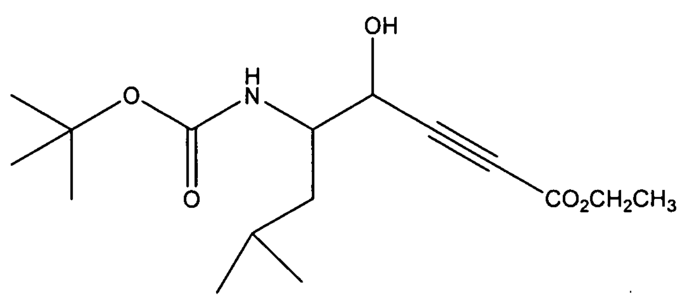
14. (Amended) The [inhibitor] compound of claim 13 having a K_i of less than or equal to 1 nM for memapsin 2.

16. (Amended) The [inhibitor] compound of claim [1] 24 which is permeable to the blood brain barrier.

17. (Amended) The [inhibitor] compound of claim [1] 24 which blocks cleavage by memapsin 2 under physiological conditions.

20. (Amended) A method of [synthesis of] preparing a Leu* Ala dipeptide isostere[.], comprising the steps of:

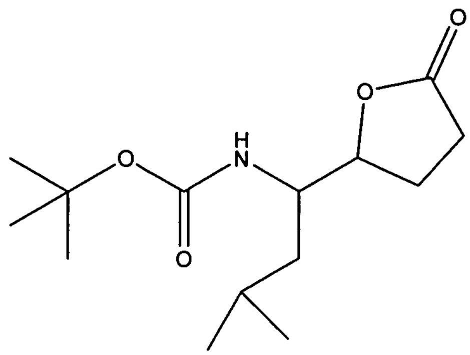
a) reacting ethyl propiolate and N-(tert-butoxycarbonyl)-leucinal in the presence of n-butyl lithium or lithium diisopropyl amine to form ethyl-5-{(tert-butoxycarbonyl)amino}-4-hydroxy-7-methyloct-2-ynoate represented by the following structural formula:



;

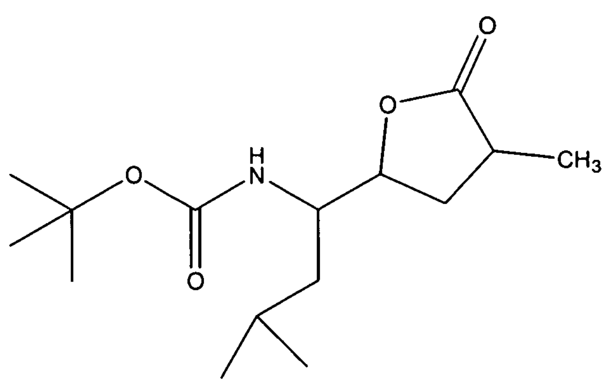
b) reacting the ethyl-5-{(tert-butoxycarbonyl)amino}-4-hydroxy-7-methyloct-2-ynoate with hydrogen in the presence of $Pd/BaSO_4$ to form an intermediate;

c) reacting the intermediate with an acid to form 5-{1'-{(tert-butoxycarbonyl)amino}-3'-methylbutyl}-dihydrofuran-2(3H)-one represented by the following structural formula:



;

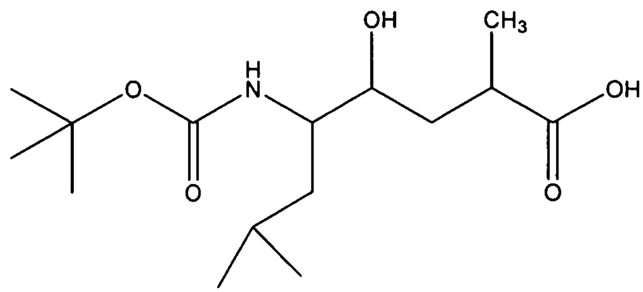
d) reacting iodomethane with 5-{1'-{(tert-butoxycarbonyl)amino}-3'-methylbutyl}-dihydrofuran-2(3H)-one in the presence of hexamethyldisilazane to form 5-{1'-{(tert-butoxycarbonyl)amino}-3'-methylbutyl}-3-methyl-dihydrofuran-2(3H)-one represented by the following structural formula:



; and

e) reacting 5-{1'-{(tert-butoxycarbonyl)amino}-3'-methylbutyl}-3-methyl-dihydrofuran-2(3H)-one with a base to form a Leu* Ala dipeptide isostere

represented by the following structural formula:



21. (Amended) A method for treating a patient to decrease the likelihood of developing or the progression of Alzheimer's disease comprising administering to the [individual] patient an effective amount of [an inhibitor of memapsin 2 having an K_i of less than or equal to 10^{-7} M or which binds to crystallized enzyme characterized by the parameters in Table 2 when bound to OM-99-2] a compound of Claim 24.

23. (Amended) The method of claim 21 wherein the inhibitor blocks cleavage of [APP] amyloid precursor protein.

Alzheimer's Alchemy

Turning an innocuous nerve-cell protein into brain-wrecking beta amyloid

By CAROL EZZELL

The protein bobs along like a buoy adrift on the nerve cell's outer membrane. Suddenly, a whirlpool beneath the surface pulls a patch of the membrane inside the cell, dragging the protein with it. The membrane fuses, closing the surface hole and leaving the protein trapped in a minuscule membrane bubble floating through the cell's watery interior.

Before long, a larger intracellular globule engulfs the tiny bubble, plunging the hapless protein — known as amyloid precursor protein — into an enzymatic hell. Powerful acids from inside the globule attack the protein where it is most vulnerable, chewing it into unrecognizable bits. Its protein dinner digested, the globule — called a lysosome — surfaces through the membrane to spew out the remains, among which is a sticky protein fragment called beta amyloid — the stuff of Alzheimer's disease.

A violent scenario like this may occur countless times each day within the brains of everyone, according to neuroscientists such as Dennis J. Selkoe of Harvard University Medical School in Boston. "All of us have the capacity to make these fragments, and in point of fact, all of us develop beta amyloid deposits with age," asserts Selkoe. "Alzheimer's is just a quantitative exaggeration of something that happens normally."

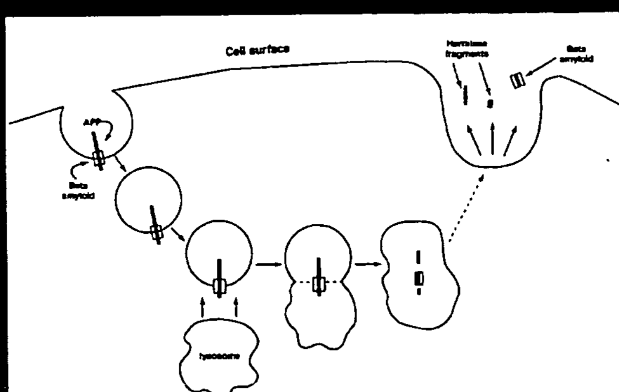
This theory — shared by a growing contingent of Selkoe's colleagues in Alzheimer's research and supported by recent studies — is causing a minor revolution among scientists studying the disease. Neuroscientists are now exploring all phases of beta amyloid production in nerve cells, looking for clues as to why some people make more of the destructive protein than others and go on to develop Alzheimer's disease. They are also turning up molecular evidence of how beta amyloid achieves its effects, laying waste to the memories and personality of an otherwise healthy individual.

Eighty-five years after its first description as a distinct malady by German neurologist Alois Alzheimer, Alzheimer's disease has become so commonplace that people joke about having it when they can't find their car keys. The National Institute on Aging estimates that Alzheimer's currently afflicts roughly 4 million Americans and projects that the disease will eventually strike half of all people who live beyond the age of 85. Although patients in the initial stages of Alzheimer's may experience only short-term memory lapses and occasional confusion, as the disease progresses they often revert to a childlike mentality and must be waited on and watched around the clock by family members or placed in a nursing home. Eventually, most Alzheimer's patients lose control of their bodily functions, and devolve into a vegetative state. Finally, some die of pneumonia, while others die in their beds from no apparent cause.

Doctors can only diagnose Alzheimer's through a process of elimination, ruling out other disorders such as a slight stroke, a brain tumor, or even an adverse drug reaction. A definitive diagnosis must await death and an autopsy, when a pathologist can view the telltale "senile plaques" that pock the brains of Alzheimer's victims.

In 1984, researchers discovered that these senile plaques consist of a central core of beta amyloid protein, surrounded by a cluster of abnormal nerve cells clogged with twisted fibers called neurofibrillary tangles. Last year, a research team led by John Hardy of St. Mary's Hospital Medical School in London found that some people with an inherited, early-onset form of Alzheimer's shared a specific mutation in their gene for amyloid precursor protein (APP) (SN: 2/23/91, p.117). Researchers have so far not determined the function of APP, which is embedded in the outer cell membrane.

Following the publication of Hardy's results — and several successive papers by other groups reporting similar findings — scores of Alzheimer's researchers began scrutinizing how APP gets converted into the fateful beta amyloid. Many became convinced that beta amyloid is an abnormal protein produced only when some biochemical process in the brain goes awry, possibly triggered by a mutation in APP. By understanding this abnormal process, these researchers reasoned, they might find a way to block beta amyloid production and treat or prevent Alzheimer's.



According to a hypothetical model, lysosomes attack Alzheimer's precursor protein (APP) after it gets pulled inside a brain cell. Enzymes within the lysosome chop APP into several fragments, including beta amyloid.

Back-to-back reports in the Feb. 7 SCIENCE challenge this notion, however. Two teams of researchers led by Steven G. Younkin of Case Western Reserve University in Cleveland describe studies showing that there are no "normal" or "abnormal" APP-degrading processes. They report that both normal and Alzheimer's nerve cells possess two separate pathways for breaking down APP — the secretory pathway, which yields apparently harmless protein fragments, and the lysosomal pathway, which carves beta amyloid from APP.

In the first study, Younkin and a group of colleagues chopped up cells taken from the brains of individuals who died of Alzheimer's and persons who died of other causes. They poured slurries of each through separate tubes filled with microscopic beads coated with special antibodies made to stick specifically to APP. The antibody-coated beads attracted the APP in the slurries but allowed all other proteins to pass through the tubes. The researchers then recovered the APP by pouring a solvent through the tubes to free the APP from the tiny beads.

When they separated the two purified APP samples on a gel according to size, they found that both contained intact

EXHIBIT

A

APP. But to their surprise, they discovered that both samples also contained five shorter APP fragments, the two largest of which contained the beta amyloid pr tein.

"This shows that amyloid isn't just produced in Alzheimer's brains," concludes Younkin.

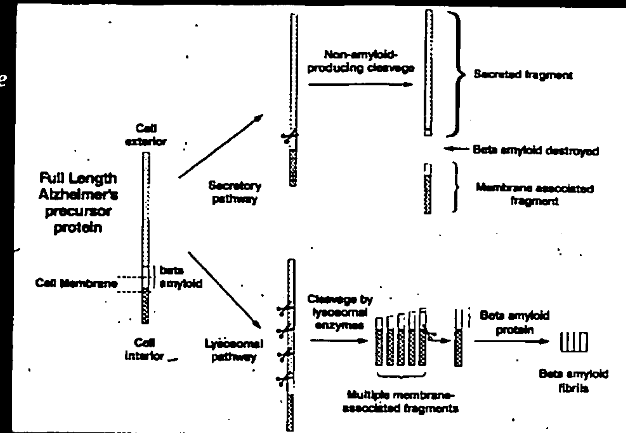
In the second SCIENCE paper, Younkin and another group – including Harvard's Selkoe – demonstrate that APP fragments with the potential to yield beta amyloid are formed within lysosomes, the scavengers of the cell. These globules of acids and enzymes usually roam the cell's interior, digesting worn-out proteins and other cellular refuse. Younkin and his colleagues treated normal nerve cells with chemicals known to block lysosomal enzymes. When they chopped and filtered the cells, they found two kinds of large APP fragments, neither of which contained the full beta amyloid protein.

Earlier research by others had shown that these two large fragments are the products of a single biochemical reaction involving an enzyme called "secretase," which clips off APP just where it pokes through the cell membrane, much like shaving off a hair. Because most of the beta amyloid portion of APP protrudes through the membrane, this process destroys beta amyloid.

Younkin asserts that both the lysosomal and the secretase pathways occur normally in healthy brains. Alzheimer's disease results, he says, when something tips the biochemical balance within nerve cells, favoring the production of small, beta amyloid-containing APP fragments over larger, harmless APP fragments.

"We're really getting very close to identifying the pathway that produces amyloid," says Younkin. "Once we've identified that pathway, then we can figure out all of the things that modify it in a way that either enhances or undermines amyloid deposition. Once we get our hands on the processing pathway and the mechanisms involved, we'll then be in a position to begin to look for drugs that block it."

Two ways for normal brain cells to break down APP. In the secretory pathway, enzymes cleave APP just outside the cell membrane, destroying beta amyloid. In the lysosomal pathway, enzymes yield five APP fragments, one of which contains beta amyloid.



Adapted from Younkin

But what steers more APP into the lysosomal pathway in some people, yielding more beta amyloid? Younkin and Samuel Gandy of Rockefeller University in New York City think the answer might rest with a ubiquitous cell enzyme called protein kinase C (PKC).

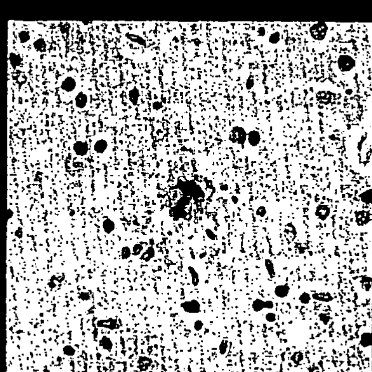
PKC – which plays a significant role in the biochemistry of learning and memory (SN: 5/25/91, p.328), among a variety of other functions – acts by tacking phosphate molecules onto specific sites on other molecules. In a study recently submitted for publication, Younkin and Gandy report evidence that the secretase pathway requires PKC in order to break down APP into harmless fragments. Younkin proposes that a slight reduction in PKC could tip the balance away from the secretase pathway, and toward the beta amyloid-generating lysosomal pathway. This reduction, he theorizes, might have a variety of causes, possibly including a mutation in the gene that directs the production of PKC.

On the other hand, neuroscientist Richard J. Wurtman of the Massachusetts Institute of Technology in Cambridge has another theory. He and colleagues at Harvard University Medical School and Boston University School of Medicine believe a membrane phenomenon they call "autocannibalism" may be to blame for steering cells onto the lysosomal pathway that leads to beta amyloid.

In the March 1 PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, Wurtman's team reports evidence that nerve cells in the brains of people who develop Alzheimer's have lower levels of key membrane molecules called phospholipids than do nerve cells in healthy individuals. One of these phospholipids, phosphatidylcholine, is also a precursor of acetylcholine, a chemical messenger between nerve cells that is reduced in the brains of Alzheimer's patients. The researchers posit that nerve cells hungry for choline compounds raid their own cell membranes for phosphatidylcholine; this leaves holes on the underside of the membrane and possibly exposes portions of APP to marauding lysosomes.

Wurtman's team is now conducting experiments seeking to determine whether such membrane defects do increase production of beta amyloid. "These are quite interesting neurochemical facts," says Wurtman. "If they can be tied together, so much the better."

Brain slice from a deceased Alzheimer's patient reveals a characteristic plaque.



Younkin

To complete the chain of events that leads to Alzheimer's, a team led by Mark P. Mattson of the University of Kentucky in Lexington has discovered how beta amyloid kills nerve cells. In the February JOURNAL OF NEUROSCIENCE, researchers working with Mattson and at Athena Neurosciences Inc. in South San Francisco, Calif., report that beta amyloid disrupts the ability of nerve cells to regulate their internal calcium levels.

Mattson's group measured calcium levels inside nerve cells grown in the laboratory after exposing them to beta amyloid. The researchers found that although beta amyloid itself produced no toxic effects, it increased the calcium influx mediated by glutamate. This amino acid normally excites nerve cells, causing them to take in calcium, but it can sometimes kill them by causing them to draw in excessive calcium amounts. Therefore, Mattson's team concluded, beta amyloid indirectly kills nerve cells by flooding them with calcium.

Fueled by developments like Mattson's, Wurtman's and Younkin's, Alzheimer's researchers will continue to focus their inquiries on beta amyloid for some time to come. "In the last five years, we've gone from 'maybe amyloid might be doing something' to pretty strong evidence that amyloid is the culprit in Alzheimer's," says Younkin. "And we're going to keep on going until we figure out how to stop it." □

Animal Models for Alzheimer's Disease

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Summary

Animal models for Alzheimer's disease are rapidly advancing with further characterization of lesions in aged primates, canines and other large animal models with the development of transgenics, having significant CNS expression of the identified genetic risk factors, APP717 and APP670/671 mutations, presenilin mutations and ApoE4. Three lines of APP transgenics have been shown to develop neuritic plaques, and 2 lines have been reported to have age-related memory deficits. Human tau transgenics have been also developed, but no clear neurofibrillary pathology has been demonstrated.

The new presenilin and ApoE4 transgenics have not yet been shown to develop AD pathology, but are being mated with other transgenics in the hopes of inducing earlier onset and more severe pathology. To date, none of these transgenics has shown quantifiable neuron loss. In vivo Aß infusion/injection approaches have produced evidence for Aß toxicity and behavioral deficits. This approach has been exploited to identify factors promoting Aß deposition and amyloid formation such as HSPGs and TGFß1. The TGFß1's effectiveness in promoting amyloid deposition has recently been confirmed in a transgenic model. Collectively, these promising animal models should allow researchers to make major strides toward therapy and prevention of Alzheimer's disease.

Key words ß-amyloid, Aß protein, transgenics, deposition, tau

Introduction

Alzheimer's Disease (AD) was originally defined as a presenile dementia accompanied by numerous neocortical senile plaques and neurofibrillary tangles and was distinguished from senile dementia of the Alzheimer type (SDAT) chiefly by age of onset. In the 1920's, these lesions were shown to have the histochemical characteristics of amyloid fibrils. Because the incidence of SDAT rises exponentially with age as does the frequency of plaques and tangles in clinically normal elderly, notably in the hippocampus and entorhinal cortex, some investigators felt that AD was an inevitable consequence of human aging. However, at late ages, the incidence curve begins to level off, and there are many examples of centenarians with no AD pathology in the regions studied. Examinations of different species of aged animals with traditional stains revealed very limited AD-like pathology in most species and no examples of any animal in which all of the features of AD were evident, leading to the prevailing view that Alzheimer's Disease, as currently defined, is a uniquely human condition. In the last fifteen years, the major amyloid-forming extracellular component of plaques has been identified as amyloid ß-protein (Aß), which is derived from ß-amyloid precursor protein, ßAPP. Heavily phosphorylated forms of the microtubule-associated protein, tau, have been identified as the major amyloid-forming component of tangles [Kosik, 1992; Joachim and Selkoe, 1992]. This has allowed sensitive immunocytochemical methods to identify deposits of these proteins in aged animal brain.

Amyloid/ Aß Deposits in Aging Animals.

Based on genetic and other evidence, amyloid deposits of Aß are centrally involved in Alzheimer's pathogenesis [Selkoe, 1996]. Animal models have been essential for understanding this pathogenesis [Price and Sisodia, 1994]. Spontaneous Aß deposit formation has been found in aged dogs [Wisniewski et al., 1970; Cummings et al., 1993], cats [Cummings et al., 1996], bears [Tekirian et al., 1996] and primates [Wisniewski et al., 1973; Martin et al., 1994; Nakamura et al., 1995; Heilbroner and Kemper, 1990]. In general, as is the case in normal human aging, diffuse deposits are relatively common, and neuritic plaques are relatively rare. In aged dogs and cats, the Aß is primarily present in diffuse deposits with little evidence for a progression to neuritic pathology defined by silver stains or altered tau immunostaining [Wisniewski et al., 1996; Cummings et al., 1996; Tekirian et al., 1996; Cummings et al., 1996]. However, altered neurites can be found in plaques in aged dog brain ultrastructurally [Wisniewski et al., 1970]. The ab-

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Alzheimer's Disease Review **is available online at http:// www.coa.uky.edu/ADReview/.** Published by the Sanders-Brown Center on Aging, University of Kentucky.

normal neurites in the diffuse deposits in the aged dog may be similar to neurites in diffuse deposits in some regions in humans and some more developed plaques in normal aged brain which also lack altered tau, but contain ubiquitin-positive dense bodies [Dickson et al., 1990]. Considerable variation in susceptibility exists between different breeds of dogs with boxers being the most susceptible. Amyloid deposits usually appear in late middle age, beginning with a very diffuse hippocampal cloud of Aß42 over the outer molecular layer [Tekirian et al., 1996; Cummings et al., 1996]. Even at late ages, there is little or no progression to neuritic plaques. In primates, dramatic differences between species exist. Squirrel monkeys develop primarily vascular amyloid [Walker et al., 1990]. Rhesus monkeys develop diffuse and neuritic plaques with less vascular amyloid [Heilbroner and Kemper, 1990; Martin et al., 1994]; lesions appear late in life beginning around age 25 and can progress to be extensive at the limits of the lifespan, around 36 years of age. Lemurs, the shortest lived primates, can develop deposits by about 9 years of age which are found associated with neurodegenerative and behavioral changes [Bons et al., 1994]. One major advantage of having the animal tissue stems from the better preservation obtained by perfusion which allows immuno-ultrastructural analysis. A very thorough and careful study of early Aß deposits in aging rhesus monkeys identified early synaptic alterations in areas of diffuse plaques [Martin et al., 1994]. The synaptic alterations and non-amyloidogenic Aß immunoreactivity in astrocytes and microglia cells and on the surfaces of and within neurons precede deposition of amyloid [Martin et al., 1994]. The early involvement of neurons is consistent with the cytoarchitectonic distribution of plaques in monkeys [Heilbroner and Kemper, 1990] and the previously discussed diffuse Aß deposition in the entorhinal projection field in the outer molecular layer of the canine hippocampus. The appearance of deposits in specific projection fields is most readily explained by a neuronal source or sink for the Aß. If neuronal surfaces are really decorated with Aß in early diffuse deposits in monkey [Martin et al., 1994] and man [Probst et al., 1991], then the neuronal surface would be a sink for Aß even if neurons are also local sources. Unfortunately, since Aß is derived from a fragment of a larger precursor which also breaks down in lysosomes, Aß sequences are present in various APP fragments and not just Aß, making cellular immunoreactivity somewhat difficult to interpret. This caveat is evident in the primate study which also found increased APP, including C-terminal APP, in similar cellular sites [Martin et al., 1994].

Collectively, the studies in aged animals suggest that diffuse plaques need not give rise to neuritic plaques (as in aged beagle dogs) and that independent factors, presumably genetic, can modulate vascular amyloid (Squirrel monkey) and primarily parenchymal (rhesus) Aß deposition. The cross- species variation also suggests that similar heterogeneity within humans may reflect the variation of processes that can proceed independently or arrest at early stages, rather than merely the interruption of a single inexorable process by the death of the patient. These data indicate that genetic factors dramatically influence plaque progression from diffuse to neuritic deposits. This may help explain why some individuals at autopsy have extensive diffuse deposits and no evidence of neurodegeneration or memory deficits. Further comparative study of genetic differences, possibly in cofactors or "chaperones", Aß metabolites, Aß receptors and Aß production by different cell types in species; developing different types of deposits may yield fresh insight into CNS deposition processes and potential targets for controlling plaque progression.

Neurofibrillary Tangles (NFT).

NFT stain as amyloid, but were shown by Kidd and Terry in the early 1960's to contain an ultrastructurally distinct type of twisted or "paired helical filaments" (PHF) quite distinct from the wispy, thinner, single filaments found in plaque amyloid. This difference in structure reflects the fact that tau protein, not the Aß found in plaques, is a principal component of tangles. Because tangles are found in dementia pugilistica [Dale et al., 1991] and have been reported in a number of different conditions [Wisniewski et al., 1979], they appear to be a special type of response to injury. None of the early investigations of aging animals revealed NFT of the PHF type found in AD. In particular, aged rodents, dogs and primates did not develop NFT [Wisniewski et al., 1970; Wisniewski et al., 1973]. Various attempts were made to induce tangles in animal models. Aluminum salts and colchicine both produce a neurofilament-immunoreactive neurofibrillary pathology [Wisniewski et al., 1982; Dahl et al., 1980], but not PHF. In general, it has been relatively easy to induce immunostaining with antibodies which recognize phosphorylated tau or neurofilaments in response to injury, but to date, none of the animals appear to develop tau amyloid and PHF. Even chronic infusion of phosphatase inhibitors such as okadaic acid which results in an accumulation of significant somatic phosphotau staining, failed to produce clear tangles [Arendt et al., 1995]. More recently tau-positive NFT have been reported in aged bears [Tekirian et al., 1996] and aged sheep [Nelson and Saper, 1995] indicating that this is not an entirely human-specific lesion. Attempts to develop more convenient rodent models, including transgenic mice expressing human tau [Gotz et al., 1995] or APP have failed to produce NFT to date, but this work is in the early stages of development. Tau is normally primarily axonal. The human tau transgenics show somatodendritic tau which labels with antibodies to phosphorylated epitopes that would be considered "pretangle" in AD brain. Phospho-tau-containing neurites have been

found associated with some plaque neurites in the oldest APP717 mutant mice [Games and Masliah, unpublished], in the APPsw mice [Hsiao et al., 1997] and in the Sandoz APP751sw transgenics driven by the Thy1 promoter [Sommer et al., 1996]. Although NFT may not develop in the single transgenics, dual transgenics expressing mutant APP and tau may succeed. Or they may not. Since aged rodents and even aged primates developing plaques do not normally develop any PHF tangles, it is currently impossible to predict what genetic elements are required for tangle formation. Beyond Aß-induced degeneration and human tau, some species-specific allele may be required for NFT formation, possibly a protein kinase or phosphatase. Despite theoretical attempts to link tangle formation to the ApoE4 allele, the critical difference is not likely to be in known ApoE alleles which differ in the presence or absence of cysteine. Non-human primates have an apoE4 -like genotype and ApoE3 appears to have developed recently in human evolution [Poduri et al., 1994; Mufson et al., 1994; Gearing et al., 1994]. The evolutionary emergence of E3, rather than E4, is correlated with the predilection for tangles of PHF in humans. However, the absence of cysteine in most animal apoE sequences does not imply that it has a human E4- like conformation or biology. In fact, recent evidence from Mahley and his collaborators at the Gladstone group argues that the Arg61 residue, which is involved in a salt bridge determining the functional differences between human E3 and E4 [Dong et al., 1994; Mahley, 1997] is not present in rodent ApoE. Therefore, rodent ApoE is more like human E3 with respect to conformation and receptor interactions.

Lesion Models

Various groups have sought to create animal models for the behavioral impairments found in AD by selectively inducing lesions in areas known to degenerate in AD brain with significant impact on known functions. This line of experimentation is bolstered by successes of regionally defined lesions induced by MPTP as a Parkinson's model and kainic acid as a possible Huntington's model. Initial reports during the 1970's of large and relatively selective cholinergic loss in the basal forebrain inspired efforts to induce cholinergic deficits based on lesions to the septal area. Behavioral deficits can be produced by lesioning septal or cholinergic neurons, and the consequences of the loss of specific cell populations can be determined [Crismon, 1994]. More importantly, some clinical benefit can be obtained with drugs targeting cholinergic losses such as tacrine [Crismon, 1994]. However, this type of model fell out of favor for three reasons: 1) it was realized that neurodegeneration in AD is widespread and many neurotransmitter systems are involved, 2) the toxin-based lesions offer the opportunity to study the consequences of particular cell loss, but did not address the fundamental causes of neurodegeneration in AD, and 3) lesions produced by colchicine [Dahl et al., 1980] or aluminum [Wisniewski et al., 1982] did not adequately reproduce lesions like those seen in AD. In particular, the "neurofibrillary tangles" with these lesions did not contain PHF or phosphorylated tau.

This approach is certainly not dead, but awaits the discovery of more relevant toxins. For example, more recently, infusions of okadaic acid have been reported to result in elevated phosphorylated tau staining and Aß-immunoreactive lesions which may contain extracellular Aß aggregates [Arendt et al., 1995].

Transgenic Mouse Models

The identification of genetic variations in AD risk factors (ApoE4) [Corder et al., 1993] and early onset, familial AD (APP670/671 and APP717 mutations [Goate et al., 1991; Mullan et al., 1992] and numerous presenilin 1 and 2 mutations [Sherrington et al., 1995; Levy-Lahad et al., 1995]) have made it possible to construct transgenic mice harboring genetic factors which promote or cause AD.

Amyloid Precursor Protein

Initial attempts to produce transgenic mouse models for AD were largely unsuccessful. Relatively low copy number (1-8), APP751 transgenics with a neuron specific enolase promoter were initially reported by Cordell and collaborators to have age-dependent Aß-immunoreactive deposits, but not thioflavin S or Congo red deposits [Higgins et al., 1995]. These APP751 mice were later shown to have memory deficits in a Y maze [Moran et al., 1995]. Similar APP695 transgenics failed to display any AD-related phenotype. Bonafide, fibrillar amyloid deposits were found by Games et al. [Games et al., 1995] in a line bearing a APP717F minigene construct driven by a platelet-derived growth factor (PDGF) promoter that has been shown to result in 4-6 fold elevations in APP mRNA over endogenous mouse levels with 80% containing the KPI insert [Rockenstein et al., 1995]. The deposits were evident in mice 6-8 months of age in hippocampus, cortex and corpus callosum. Some plaques had thioflavin S-positive cores and plaques with abnormal neurites and fibrillar cores were demonstrated ultrastructurally. Qualitative evidence of synapse/ synaptophysin loss was present in the hippocampus. Homozygotes have shown spectacular amyloid deposition at 20 months. More complete neuropathological descriptions, particularly from E. Masliah's work on the mice presented at meetings suggest that the mice show neurodegeneration including some plaque neurites positive for altered tau, but no tangles. These mice are clearly a useful model for studying Aß secretion, deposition and plaque pathogenesis and represent a major advance. The reasons for the success with these "Athena/ Exemplar" mice have been suggested to in-

clude the use of the PDGF promoter, very high level expression of APP, the 717 FAD mutation and the use of the minigene construct which resulted in 80% KPI-containing APP.

More recently, 2 lines of APP transgenics have been developed by K. Hsiao. The first line, in the FVB mouse strain background, showed that the expression of different APP695 constructs driven by the prion promoter resulted in a gene dose-dependent syndrome of neophobia, seizures, early death and reduced glucose utilization that resembled a phenotype developed in a small percentage of non-transgenic FVB mice with advanced age [Hsiao et al., 1995]. The high expressing mice died prior to 6 months of age and no amyloid plaques were observed. A more recent line of mice from Hsiao using human APP695sw with the 670/671 "Swedish" double mutation driven by the prion promoter (Tg2576) expressing at 3-5 times the endogenous APP levels have shown very clear age-related amyloid deposition and grossly elevated brain Aß concentrations accompanied by memory deficits [Hsiao et al., 1996]. These mice also appear to have neuritic plaques with reactive astrocytes and microglia by 12 months and can live for at least 18 months [Hsiao et al., 1996; Cole et al., 1996]. They demonstrate that the PDGF promoter, KPI domain or 717 mutation or minigene construct are not essential to develop significant AD pathology. A third APP transgenic mouse model developed by B. Sommer and colleagues at Sandoz using the Thy1 promoter to drive APP751sw also shows typical amyloid plaque pathology by one year of age [Sommer et al., 1996]. The critical common parameters appear to be the high level expression of Aß which is around 40-50pmol/ gm wet weight in the successful mice and survival to at least 6-8 months for development of the deposits. There is no doubt that like the Athena mice, the Tg2576 mice from Hsiao and the Sandoz mice will be useful model systems for looking at factors regulating Aß production and deposition. Survival in APP transgenics with high levels of expression is curiously strain-dependent as Hsiao has found that the successful Tg2576 transgene results in the FVB neophobic early death syndrome when bred into the FVB or pure C57Bl background [Hsiao et al., 1997]. Intriguingly, transgenics expressing a murine APP695 construct with a double mutation in the a secretase site (dramatically reducing APP secretion) driven by the neuron specific elements of the Thy-1 promoter resulted in a 3-fold transgene APP over endogenous APP and a similar neophobia and seizure-prone, early death phenotype with no Aß deposits in pure FVB and C57Bl host backgrounds [Moechars et al., 1996]. These mice also showed necrosis, apoptosis and astrogliosis in cortex, amygdala and hippocampus. A similar phenotype was not found in APP751 mice with the same construct with apparently similar high levels of APP expression. At the recent Keystone meeting on Alzheimer's (Feb. 97), it was reported by Hsiao and Borchelt that crossing the neophobic APP FVB mice with SOD transgenics markedly increased longevity [Hsiao et al., 1997] . The effect was clear despite the complications of background strain effects in the cross on lifespan. Seizures, neurodegeneration and apoptotic cell death were also observed in transgenic mice expressing only the Aß domain without, but not with, a signal peptide sequence, implying that cytosolic overexpression and accumulation of Aß could be neurotoxic [LaFerla et al., 1995]. However, since cytosolic accumulations of Aß are not evident in AD, the results are instructive, but the model doesn't resemble AD with respect to known Aß compartmentalization. While this model shows gross neuron loss at early ages, there is no quantitative data to show major neuron loss in the amyloid plaque forming animals at the ages examined. Quantitative neuron counts by Hyman's group in susceptible regions in AD such as entorhinal cortex have so far failed to reveal significant neuron loss in the Hsiao mouse at 1 year or in the Athena mouse at 18 months [Hyman, 1997]. Whether neuron loss develops at late ages or fails to develop in these mice because of species differences or protective effects of the transgene remains unknown.

Since very high APP expression level has been necessary to produce Aß deposits, but is not found in AD brains, it is important to consider the consequences of overexpression of APP per se which appears to produce a remarkable strain-dependent phenotype in the absence of Aß deposition. APP695, but not APP751 overexpression in the absence of Aß deposition has been reported to accentuate synaptic loss after injury [Mucke et al., 1996]. APP knockout mice exhibit reactive gliosis and neurological deficits suggesting neuronal injury [Zheng et al., 1995] consistent with in vitro studies showing important functional roles for APP including support of neuronal process growth [Qiu et al., 1995; Roch et al., 1993; Small et al., 1994]. Overexpression of the functional domains of APP may have a significant impact on the phenotype independent of Aß production which may not resemble AD at all. Furthermore, overexpression of the transgene may actually decrease endogenous murine APP synthesis shifting the balance between KPI and 695 forms. If these two forms compete for transport mechanisms through common domains such as the Aß domain implicated in axonal transport by Beyreuther [Osaka,1996], then marked overexpression of one form may further reduce the availability of the other in neuronal processes. For example, marked overexpression of APP695 might reduce serine protease inhibitor activity in axons and synapses. Protease nexin I, another serine protease inhibitor was recently reported to inhibit apoptotic motor neuron death during development and after axotomy [Houenou et al., 1995]. If the KPI domain is more neuroprotective in vivo, then the Athena/ Exemplar mice may be neuroprotected relative to the APP695 transgenics, particularly in strains with lower ratios of serine

protease inhibitor to protease activity. Similarly, overexpression of other, non-KPI functional domains of APP may alter CNS function, and any of these domains could have a major neurobiological impact. For these reasons, it is too early to tell whether any specific behavioral or neurodegenerative problem observed in the Athena or Hsiao mice derives from overexpression of Aß or other APP domains. Because it is certainly possible, if not likely, that aspects of the phenotype in APP transgenics involves non-Aß domains, other approaches to increase Aß in vivo are clearly warranted.

ApoE

ApoE4 is the major identified genetic risk factor for AD [Corder et al., 1993]. ApoE transgenics have been made for studies of atherosclerosis. However, the mechanism behind ApoE4 's role in AD pathogenesis has not been unambiguously established in vivo. Transgenics expressing human ApoE alleles in the brain are under development in several laboratories using a variety of cDNA and genomic DNA approaches. An initial paper characterizing lines of genomic human ApoE2, E3 and E4 transgenics in an ApoE knockout background which use the native promoter and 3' enhancer elements has been recently described [Xu et al., 1996]. Some of these lines show high levels of expression in the brain and should be valuable tools for establishing human ApoE isoform dependent CNS effects relevant to differential risk for Alzheimer's disease. The human ApoE transgenics show ApoE immunoreactivity in a subset of cortical neurons intriguingly similar to primate, but not rodent brain.

Presenilins

Presenilins were recently identified as homologous multiple membrane spanning proteins containing mutations in chromosome 14-linked (PS-1) and chromosome 1- linked (PS-2, Volga German) early onset FAD families [Sherrington et al., 1995; Levy-Lahad et al., 1995]. While the function of presenilins remains unknown, there is fairly convincing evidence that, like the APP717 mutations, they result in an increased percentage of the longer, more amyloidogenic form of Aß, Aß42 [Scheuner et al., 1996]. PS-1 transgenics with the A246E mutation have been reported, but the mice have not shown detectable neuropathology out to 8 months of age [Thinakaran et al., 1996]. Pathology may develop later or in PS-1 FAD transgenics or in crosses with transgenics expressing human APP. Karen Duff has presented data to show that PS-1 transgenics crossed with Hsiao's Tg2576 APPsw mice have increased Aß42 levels, but data on pathology require further study [Duff, 1997]. Presenilin knockout mice have been constructed by several labs and the recently presented work reveals the homozygous PS1 null mice have lethal developmental defects in axial somatic segmentation and neurogenesis [Wong et al., 1997]. The heterozygotes with a single functional PS1 gene develop normally and might prove useful for AD research if PS AD mutations involve a loss a function as suggested by elegant experiments from Haas and others with C. elegans.

Tau proteins

Although no tau mutations have been identified in FAD, neurofibrillary tangles represent a significant component of AD pathology which could be highly desirable to study in an animal model. Recently, human tau transgenic mice have been constructed which express human tau in CNS neurons and show somatodendritic phosphorylated tau, but do not appear to make spontaneous NFT at the ages examined to date [Gotz et al., 1995].

Aß Injection/ infusion models

In culture systems, large amounts of Aß can be readily added directly to neurons with resulting toxicity. Aggregation of Aß is generally believed to be a critical factor regulating toxicity in vitro [Pike et al., 1993] and it may be of critical importance in vivo as well. Attempts to reproduce these results by in vivo injections have met with varying results [Kosik and Coleman, 1992]. In general, injection of small, nanogram amounts of soluble Aß does not appear to produce significant short term neurotoxicity. A major difficulty encountered in attempting to inject larger, microgam amounts is the tendency to aggregate and the toxic solvents used to prevent aggregation in the test tube, syringe or pump. The first published effort to demonstrate neurotoxicity in vivo used 10-15 µg of Aß in the HPLC solvent (35% acetonitrile, 0.1% TFA) used to purify the Aß [Kowall et al., 1991]. We later replicated this experiment, confirming the Aß toxicity and demonstrating that the toxicity depended on the solvent used; the control with the same solvent and reverse peptide had a smaller cavity at the injection site [Waite et al., 1992]. Independently, we had injected preformed amyloid from intact and sonicated plaque core preparations and control lipofuscin preparations isolated from human brain into rat brains and observed persistent Aß immunostaining, apparent neurotoxicity and a massive microglial response [Frautschy et al., 1991; Frautschy et al., 1992]. These experiments were followed by numerous attempts to explore the response to injection or infusion of synthetic Aß into animal brains [Kosik and Coleman, 1992; Podlisny et al., 1993]. The results vary widely and seem to depend on the details of the experimental protocol used. In general, injection of small amounts of soluble Aß was not neurotoxic, but larger amounts or preformed aggregates were. If Aß is injected as aggregates (due to large amounts, salt or preaggregation, or HSPG or other cofactors promoting ag-

gregation), then one can easily see Aß deposits or amyloid, microglia, and reactive astrocytes in the region around the tip of the needle tract or around the ventricle if ventricular infusion is used.

Injection of a small amount of soluble Aß (500ng) directly into hippocampus resulted in no behavioral deficit above that seen with vehicle alone [Winkler et al., 1994] but intraventricular infusion of 100µg of Aß produced behavioral deficits [Nitta et al., 1994]. Behavioral deficits have also been reported after 15 daily intrahippocampal injections of 2 µg Aß1-40 into cannulated rats [Cleary et al., 1995]. In agreement with data from Snow's group, which found that HSPGs were needed to get good Aß deposited as amyloid [Snow et al.,.1994], we have found that infusion of soluble Aß1-40 usually results in very little Aß deposition. This may be explained by recent unpublished experiments in our laboratory which indicate that injected, soluble Aß is rapidly cleared from cortex or hippocampus, an observation which would help explain the apparent lack of toxicity of low levels of soluble Aß in vivo. Chiefly to avoid problems caused by aggregation within the pump or massive aggregation at the tip of the needle tract where injection trauma is a major confounding variable, we have recently introduced a two site injection/infusion paradigm where soluble Aß1-40 is infused into the rat ventricle, and modifying factors which might promote deposition are injected at another site away from the needle-tract. Using this system, we found that a single injection of TGFß1 with Aß1-40 infusion resulted in large numbers of widely-dispersed, thioflavin S positive Aß deposits [Frautschy et al., 1996]. This appeared to reflect an inhibition of microglial clearance of Aß which occurred in the absence of co-factor injections, but TGFß has several effects which could contribute to Aß deposition. Based on Snow's data with HSPG infusions, the well established TGFß mediated increases in extracellular matrix proteins like HSPG would be a strong candidate mechanism. A similar amyloidogenic effect has been shown by Mucke with a transgenic line overexpressing TGFß1 around vessels which develops spontaneous vascular thioflavin positive Aß deposits [Mucke et al., 1997]. This confirms that TGFß1 is one physiologically relevant amyloidogenic factor known to increase after injury which can play a significant role in injury associated risk. It also illustrates the utility of our two site injection/Aß infusion approach. Using the two site model and quantitative immunocytochemistry and Aß ELISA, we are currently evaluating the effect of a number of injected cofactors on deposition of infused Aß1-40.

Aß injection/infusion models can be used for evaluating drugs which target Aß aggregation or deposition (as opposed to production), neurotoxic effects or a microglial/inflammatory response. They have two major disadvantages. First, a penetrating brain wound is currently required to get adequate Aß into the brain; so everything is superimposed on a focal traumatic injury. Second, controlling aggregation raises a number of problems in getting large amounts of Aß dispersed into the brain away from the site of injury. Intraventricular pumping of Aß1-40 in salt free buffer is one solution. Putting in significant amounts of the more rapidly aggregating Aß1-42, quite possibly the critical pathogenic Aß species, has been a difficult task. However, widely dispersed, diffuse Aß1-42 deposits accompanied by significant neurodegeneration was recently reported by Frautschy and colleagues in rat brains ventricularly infused with Aß1-42 using high density lipoprotein as a carrier [Frautschy et al., 1996]. In this study, thioflavin S positive deposits were not observed, and the majority of CNS Aß assayed by Sandwich ELISA at the end of the 30 day infusion was soluble, that is extractable in aqueous buffer. This neurotoxicity of relatively low doses of "soluble" Aß42 with a lipoprotein carrier in vivo needs to be carefully confirmed. A quantitative demonstration of the neurotoxicity of small amounts (300pg) of preformed Aß fibrils injected into aged monkey brain has recently been presented by Geula and Yankner [Geula et al., 1996]. The aged primates appear to be more sensitive to neurodegeneration than rodents which may account for the lack of gross neuon loss in transgenic animals with extensive Aß deposits. These studies appear to confirm the potential neurotoxicity of Aß in vivo and suggest that injection or infusion studies may also have utility in modeling Aß- induced neuron loss.

Conclusion

The lack of an adequate animal model for AD has been a serious stumbling block in understanding the disease and developing therapeutic drugs. Identification of genes and mutations relevant to AD pathology has opened up the opportunity to make transgenic mice harboring identified risk or causal factors for AD. The first efforts focused on overexpression of APP and then FAD mutant APP transgenes and eventually resulted in the production of mice which develop significant AD pathology with age. They are undoubtedly good models for exploring therapies targeted at Aß secretion, deposition, aggregation and probably the inflammatory response. The extent and causes of the neurodegeneration and behavioral deficits seen in these models require additional study and may well involve high expression of non-Aß APP domains. Transgenics with the presenilin, apoE4 and tau genes are continuing to be developed. It is too early to know whether they will also result in significant AD pathology or can be successfully mated to the APP transgenics to produce superior models. With growing evidence that Aß plays a critical pathological role in the

disease, models with direct injection or infusion of Aß would also appear to be useful for identifying factors that promote Aß deposition and studying neurotoxic and behavioral effects of Aß. The next several years should see the development of compounds which clearly ameliorate AD pathology or behavior in rodent models leading to new therapeutic approaches for treating this tragic, devastating disease.

Acknowledgments

This project was funded by NS30195 (SAF), AG10685 (SAF), VA Merit Grant (SAF, GMC), AG9009 (GMC) and AG11125 (GMC). We are grateful to the Thomas E. and Elizabeth Plott family for their support. We acknowledge the helpful comments of Dr. Marni Harris, Dr. Bruce Teter and Jason Sigel in reviewing the manuscript.

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